THE EFFECTS OF FREEZE-DRIED WATERMELON ON BONE AND CLINICAL PARAMETERS OF OVARIECTOMIZED MICE

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Bachelor of Science in Nutrition

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Lisle, IL

2011

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
May, 2014

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ON BONE AND CLINICAL PARAMETERS OF

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ACKNOWLEDGEMENTS

This thesis would not have been possible without the immense support and help of my advisor Dr. Edralin Lucas. Dr. Lucas always took out her valuable time to provide her advice and assistance with every aspect of my graduate degree. She allowed me to be involved with numerous research opportunities and the knowledge I gained is invaluable. Her efforts to make me successful will resonate throughout my career and will never be forgotten.

I would also like to thank my thesis committee members Dr. Brenda Smith and Dr. Stephen Clarke. Dr. Smith continuously provided me with her expert opinion on bones and pointed me in the right direction when I was unsure of what to do next. She has been essential in my professional development as a writer and a public speaker. Dr. Clarke was always available and patient when I needed help with any experiment or with understanding difficult concepts. He always made sure that I fully understood before moving on and would never hesitate to come in the lab and work on an experiment with me.

I also want to express my gratitude to all of my peers and the amazing Nutritional Sciences faculty and staff that has helped and encouraged me during this process. I would especially like to thank Julie Huber and Carol Bier for bringing me to Oklahoma State University and providing me with a great dietetic internship experience. Without their support, this graduate degree would not have been possible.

Finally, I am forever indebted to my parents for providing me the opportunities and environment to get the best education and experiences, and for supporting my ambitions, physically, emotionally, and financially. Without them I would not be who and where I am today and there will never be enough words to express my gratitude. I would also like to thank my brothers and sisters, Aamir, Nabiha, Shireen, Younus, and Taha, for continually being there to encourage me, my beautiful nieces Laila and Zahra for always making my day better no matter how stressful, and my loving grandparents for their unceasing prayers for my success.

Name: MARYAM MAHMOOD

Date of Degree: MAY, 2014

Title of Study: THE EFFECTS OF FREEZE-DRIED WATERMELON ON BONE AND CLINICAL PARAMETERS OF OVARIECTOMIZED MICE

Major Field: NUTRITIONAL SCIENCES

Abstract: The role of fruits and vegetables in the prevention and treatment of chronic diseases such as osteoporosis are continuously being explored. The purpose of this study was to investigate the dose-dependent effects of freeze-dried watermelon in the prevention of bone loss in ovariectomized (OVX) mice, a model of post-menopausal osteoporosis. We *hypothesized* that compounds in watermelon such as lycopene and citrulline would help prevent bone loss in this animal model. Three month old C57BL/6 female mice (n=68) were sham-operated or OVX and randomly assigned to treatment groups for 12 weeks: sham-control (SHAM), OVX-control, OVX + 1%, 10% or 25% (w/w) freeze-dried watermelon (WM), or OVX-control with alendronate (OVX-ALN) injection (100 μg/kg body weight). All diets were isocaloric, isonitrogenous and had the same calcium and phosphorus concentrations. The 25% WM group had significantly higher final body weight and % body fat compared to the OVX-control group. The DXA scans using PIXImus showed watermelon was not able to prevent the decrease in whole body, tibial, and lumbar bone mineral content and density due to estrogen deficiency. Micro-computed tomography (μCT) analyses showed no similarities between WM-fed groups and SHAM for tibial trabecular and cortical bone microarchitecture. However, the lumbar trabecular micro-architecture analyses revealed that the WM 10% group had similar connectivity density to the SHAM group and higher than the other two doses of WM and OVX-control groups. Bone volume, structure model index and trabecular separation of the WM 10% group are similar to the SHAM and OVX-control groups. Watermelon supplementation increased liver catalase mRNA and tended to increase plasma glutathione peroxidase activity. Our data indicates that watermelon, in a moderate amount, might be a suitable dietary option for maintaining skeletal health.

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CHAPTER I

INTRODUCTION

Osteoporosis is a metabolic bone disease, which increases the risk of bone fractures as bone demineralization progresses and is classified as a major public health problem. Overall, 200 million people in the world have osteoporosis and it has been linked to hormonal and other age related physiological changes. It can affect both men and women but it is estimated that 30% of all postmenopausal women in the United States and European Union have osteoporosis (1). Research shows that the withdrawal of estrogen post menopause can decrease the rate of bone formation and anitresorptive drugs and intermittent PTH are commonly used as treatments (2,3). In 2005, total costs related to osteoporosis neared 19 billion dollars in the U.S and is projected to increase by 50% by 2025 (4). The most common sites affected by osteoporosis include the spine, hip and wrist, followed by humerus and ribs. Osteoporosis is commonly diagnosed after the patient has had their first fracture and therefore, more focus recently is placed on determining the risk and preventing the disease (5).

The human skeleton consists of mainly two types of bone: about 80% cortical bone and 20% trabecular bone. Cortical bone is the dense solid part that surrounds the marrow spaces while the trabecular bone is described as a honey-comb like structure that fills the marrow space. The outer surface of the bone is referred to as the periosteal and the inner surface is the endosteal surface. As a person ages, their bones increase in diameter due to increased bone formation

on periosteal surface and the marrow space expands due to increased bone resorption on the endosteal surface. Along with the effects of age on the skeletal system, estrogen withdrawal due to menopause causes an increase in bone loss, which is the major contributor to postmenopausal osteoporosis. The cells that are responsible for this physiological activity of the bone are called osteoblasts and osteoclasts, and osteocytes. Osteoblasts and osteoclasts are associated with bone formation and bone resorption, respectively while osteocytes are mature osteoblasts that make up majority of skeleton, and are mechanosensory cells involved in the regulating bone turnover (6,7).

Postmenopausal osteoporosis results when there is an imbalance in the activity of osteoblasts and osteoclasts due to the decrease in estrogen production. There are multiple proteins such as receptor activator of nuclear factor-κB (RANK), receptor activator of nuclear factor-κB ligand (RANKL), macrophage colony stimulating factor (M-CSF), and osteoprotegerin (OPG) that influence osteoclast differentiation and activity and are affected by the removal of estrogen (5). Osteoclast activity is stimulated through RANKL. RANKL, a member of the tumor necrosis factor receptor (TNFR) superfamily, is manufactured in osteoblasts and bone marrow stromal cells (T and B lymphocytes) and interacts with its receptor, RANK, on osteoclasts and hematopoietic precursors cells to mediate cell resorption by controlling the transcription of DNA for cell differentiation and resorption (5,8). Macrophage colony-stimulating factor (M-CSF) and RANKL are the essential cytokines required for osteoclast formation under basal conditions (9). M-CSF, secreted by osteoblasts, interacts with its receptor c-Fms and stimulates proliferation of preosteoclasts while RANK found on progenitor cells bind to RANKL to produce mature multinucleated osteoclasts and also act on osteoclasts to increase bone resorption (5). Osteoprotogerin (OPG) is part of the TNF superfamily and is a soluble decoy ligand for RANK that inhibits osteoclasts activity (10). OPG originates from many cells including osteoblasts, heart, liver, kidney and spleen. The ratio of RANKL to OPG is one of the factors that determine the osteoclasts activity (11).

There are several factors that may influence the levels of RANKL and OPG including hormones, growth factors, inflammatory cytokines, and genetic polymorphisms, and vitamins such as Vitamin D. Estrogen has been linked to increased OPG and decreased RANKL secretion (12). Studies have shown that chronically elevated parathyroid hormone (PTH) increases bone resorption in a dose-dependent manner (13). Growth factors that may play a role in regulating bone formation and resorption include insulin-like growth factor 1 (IGF-1) and transforming growth factor-β (TGF-β). Cytokines are also major pathway regulators of the RANKL/OPG system. Some of the cytokines that promote bone resorption include interleukin 1 (IL-1), IL-6, IL-7, IL-17, and TNF-α. On the other hand, cytokines IL-4, IL-13 and interferon-γ are associated with suppressing the formation of osteoclasts (14). Estrogen deficiency promotes TNF producing T-cell activation in the bone marrow and thereby causing an increase in TNF-α production (9). TNF-α increases RANKL production and increases the responsiveness of osteoclasts precursors to RANKL. This cytokine also increase osteoclast activity and inhibit osteoblast production (15). Inflammatory cytokines can also trigger the production of reactive oxygen species (ROS), which can increase the transcription of RANK and thereby increase osteoclast formation and activity and decrease osteoblasts by promoting apoptosis (16). Together inflammation and increase oxidative stress lead to the progression and exacerbation of osteoporosis unless there is a treatment intervention.

Common drug treatment options available for osteoporosis include bisphosphonates and intermittent PTH. Bisphosphonates that work by decreasing osteoclasts-related bone resorption include drugs such as alendronate, risedronate, ibandronate, and zoledronic acid (17). However, these treatments have low compliance rates as well as unpleasant side effects including nausea, abdominal pain, esophagitis and esophageal ulceration (3). Hormone replacement therapies (HRT) have been linked to increased blood clots, heart disease, and breast and endometrial cancer (18). According to a follow up study, 1,000 postmenopausal women, the use of HRT declined by

12% from 2003 to 2004 (19). 1 in every 4 women using hormone replacement therapy, and 1 in every 5 women using alendronate, discontinued its use due to side effects including breast tenderness, menstrual bleeding, and hot flashes for hormone replacement therapies and GI distress for alendronate (18).

To reduce the effects of, or prevent osteoporosis, it is important to decrease the inflammatory and oxidative stress pathways that lead to the increased bone resorption and decreased osteoblast number and activity. Fruits and vegetables are beneficial in maintaining skeletal health is because they contain bioactive compounds that have anti-inflammatory and antioxidant properties (20). Among the variety of fruits, watermelon because of its active compounds, may play a role in maintaining skeletal health (21). Some of the bioactive compounds in watermelon include vitamin C, β-carotene, potassium, phenolic compounds (e.g. flavanoids, carotenoids, and triterpenoids) and citrulline. Lycopene is a well-known antioxidant and recent studies suggested that lycopene may actually increase osteoblast proliferation and decrease PTH stimulated osteoclast development and proliferation (22,23). Studies with postmenopausal women demonstrated that those who took lycopene supplements had much lower serum oxidative stress (such as catalase and superoxide dismutase) and bone resorption markers (such as crosslinked N-telopeptide of type I collagen) (24,25,26). An analysis of the Framingham osteoporosis study showed a relationship between increased carotenoid, such as β-carotene, and lycopene intake and reduced risk of hip fracture (27). In addition to lycopene, watermelon contains carotenoids and vitamin C as well as the amino acid (more concentrated in the rind) citrulline, which is converted into arginine in the body. The demand for arginine is increased when macrophages respond to inflammation, therefore increasing the body's physiological needs, which can be provided through metabolism of citrulline (28). Watermelon may help in the prevention of inflammatory-related conditions such as osteoporosis by providing citrulline, which can be converted to arginine.

Although lycopene, vitamins C, β-carotene as well as citrulline have been shown to have anti-inflammatory and antioxidant properties, we are not aware of studies investigating the effects of freeze-dried watermelon fruit on skeletal health and clinical parameters. Therefore, the ***objective*** of this study was to determine the extent to which watermelon would be able to prevent bone loss and attenuate clinical parameters, such as glucose and lipids, in ovariectomized mice, an animal model for postmenopausal osteoporosis. Due to the antioxidant and anti-inflammatory properties of the bioactive compounds in watermelon, our ***null hypotheses*** are that freeze-dried watermelon dose-dependently will have no effects on:

1. bone mineral density,

2. bone microarchitecture,

4. clinical parameters (i.e. glucose and lipids), and

5. antioxidant and anti-inflammatory markers.

CHAPTER II

REVIEW OF LITERATURE

Bone Function and Structure

The skeletal system is designed to provide a variety of important functions. Some of these functions include structure, movement, protection, mineral homeostasis, acid-base balance, a reservoir of growth factors and cytokines, and hematopoiesis, as well as serving as an important immune function regulator (5,6). All of these vital roles are what makes it extremely important to keep the skeletal system healthy.

The human skeleton is primarily comprised of two major types of bone, cortical and trabecular. Cortical bone that makes up about 80% of all bone is the dense solid portion of the bone that surrounds the marrow space. Trabecular bone is a network of plates and rods within the marrow space that creates a honey-comb like appearance. The cortical bone is made up of the periosteal and endosteal surfaces. The periosteal surface is the outer part of the cortical bone where bone formation is greater than bone resorption. It is a network of fibrous connective tissue that contains blood vessels nerve fibers, osteoblasts and osteoclasts, and can be found on all bones except at joints. The inner endosteal surface of the cortical bone that surrounds the marrow space and trabecular bone, undergoes more bone resorption than bone formation (6). This change in bone structure leads to the increases in bone diameter and expands the marrow space with age, increasing the risk of developing osteoporosis and fractures.

Bone Remodeling

At the cellular level bone is composed of three major cells, osteoblasts or bone forming cells, osteoclast or bone resorbing cells and osteocytes, which are mature osteoblasts. For bone remodeling to occur there must be communication between these three cells also known as "bone coupling". Bone remodeling is an intricate process that involves osteoclasts resorbing bone at a particular location and osteoblasts forming new bone over that area. Osteocytes are thought o be involved in initiating the bone remodeling process as well as terminating it (40). The health of the bone is a direct consequence of the communication between the three cells to maintain a balance in bone formation and resorption. At any given moment, 20% of trabecular bone is constantly undergoing remodeling in the body (29).

Osteoclast Function

Osteoclasts are formed from hematopoietic precursor cells that are stimulated to differentiate by binding of RANKL to RANK and the presence of M-CSF. Osteoblasts secrete RANKL, M-CSF as well as OPG, a decoy receptor for RANKL that inhibits osteoclastogenesis. RANKL is also secreted in large quantity by activated T-cells that also produce TNF-α in abundance. TNF-α binds to its receptors, p55 and p75, on osteoclasts as well (30). The binding of RANKL to RANK, M-CSF to c-Fms, and TNF-α to p55/p75, on the osteoclasts and osteoclasts precursors stimulates a cascade of intracellular events. This includes the mobilization of TNFR-associated factor proteins (TRAF), which leads to the activation of NF-κB, a transcription factor, and also c-Jun NH_2-terminal kinase (JNK), thus increasing expression of genes needed for osteoclastogenesis (31). It is not understood how osteoclasts recognize the sites that need to be resorbed however it has been suggested that osteocytes are responsible for sensing micro fractures in the bone and send signals to the osteoclasts progenitor cells (30, 40). Osteoclasts travel to the site that needs to be resorbed and attach to the surface using their extremely agile

cytoskeleton and β1 integrins to form a barrier from the bone to be resorbed and the outside environment (30). Osteoclasts create a tightly sealed acidic environment using a polarized vacuolar proton pump**,** H^+–adenosine triphosphatase bringing it to a pH of -4.5 (5,32). The acidic environment first causes the bone to demineralize leaving the organic component to be degraded by cathepsin K, a lysosomal protease released into the extracellular space on the apical side. The osteoclasts endocytose the degraded products then release it on its basolateral side (33). The degraded products released include insulin-like growth factor (IGF) and transforming growth factor-β (TGF-β), which will draw in the osteoblasts to form new bone (5).

Osteoblast Function

Osteoblasts are formed from pluripotent mesenchymal stem cells located in the bone marrow (34). Differentiation is stimulated by runt-related transcription factor 2 (Runx2), an important transcription factor that form preosteoblasts. In the presence of osterix (OSX), another important transcription factor, the preosteoblasts turn into mature osteoblasts (35). Alkaline phosphate (ALP), a protein required for mineralization of bone, and type 1 collagen are needed before bone formation takes place (36,37,38). At the molecular level, one of the most important factors regulating osteoblast differentiation is dependent on the stimulation of the canonical Wnt/ β-Catenin pathway (39). When the Wnt family of protein associate with their receptors, mainly Frizzled and (LDLR)-related protein 5 (LRP5), it sets off a signaling cascade that allows β-Catenin to be dephosphorylated and inhibit glycogen synthase kinase 3β (GSK-3β) activity, the main kinase responsible for phosphorylating β-Catenin . The dephosphorylation of β-Catenin allows it to translocate into the nucleus and promote the transcription of genes responsible for osteoblastogenesis (39). After successful differentiation, osteoblast move into the lacunae formed by osteoclasts. In the presence of mineralization factors such as vitamin D, calcium and phosphate, osteoblasts start to release components for the extracellular matrix that includes type I

collagen and other non-collagenous proteins, such as osteocalcin, osteonectin, and osteopontin (37,39).

Osteocyte Function

Once the osteoblasts have rebuilt the resorbed area, they transform into bone cells, or osteocytes, which account for about 90% of all bone cells (5). Osteocytes are thought to be the major bone cell that responds to mechanical loading. Increased mechanical load has been suggested to stimulate bone formation and decreased load stimulates bone resorption. These cells have unique dendritic processes that allow them to communicate with each other and other cells in the bone (40). Osteocytes are able to regulate osteoclastogenesis through expressing increased RANKL on their dendritic processes (41). Additionally, osteocytes are capable of regulating osteoblastogenesis by releasing sclerostin, which inhibits the Wnt/ β-Catenin pathway discussed earlier (42). The completion of the bone remodeling process leads to new bone if there is a balance between the functions of the bone cells. When estrogen is removed as in the case of a postmenopausal woman, these processes are altered leading to the development of osteoporosis.

Osteoporosis

Osteoporosis is a major health concern as the resulting fractures lead to a reduced quality of life, a decline in independence, and maintenance of overall health. Osteoporosis places a great economic burden on healthcare costs. It is estimated that by 2020, greater than 14 million people will have osteoporosis in the United States (4). Along with this, in 2005, the estimated cost of osteoporosis in the U.S was between 13.7 to 20.3 billion dollars and this has only been projected to increase (43). Costs were substantially increased in women with increasing age with 89% of costs belonging to women over 65 years of age (4). A study in 1997 by Hoerger and colleagues (44) of women over the age of 45, found that postmenopausal osteoporosis accounted for 12.9 billion dollars in total healthcare costs. The most common fracture sites in this disease include

vertebra, hip, wrist and pelvic bones (5). Vertebral fractures accounted for 27% of total fractures from osteoporosis followed by wrist (19%), hip (14%), and pelvic (7%); however, hip fractures produced the most health care costs (4).

The World Health Organization (WHO) has developed diagnostic criteria for osteopenia and osteoporosis. The classification of the disease is relative to the average BMD of a 30-year-old adult, and the severity depends on the number of standard deviations away from the reference value, also called a T score. “Normal” is defined as a T score of greater than -1.0, osteopenia is between -1.0 to -2.5, osteoporosis is below -2.5, and established osteoporosis is defined as a score below -2.5 plus a fragility fracture. A Z-score, which matches for age and gender, can also be used to diagnose postmenopausal osteoporosis (45).

Treatment Options

Pharmacological treatment options available to women inflicted with postmenopausal osteoporosis include drugs that have antiresorptive effects and also drugs that have anabolic effects. Dunosumab, bisphosphonates, calcitonin, and estrogen agonists/antagonists are classified as antiresorptive drugs. Bisphosphonates include the drugs alendronate, ibandronate, risedronate, and zoledronic acid. Raloxifene is an estrogen agonist/antagonist that is not a hormone, but provides the beneficial effects of estrogen. Treatments that have an anabolic effect or those that stimulate bone formation include teriparatide parathyroid hormone, which is a recombinant PTH hormone fragment (46).

Bisphosphonates work to decrease bone resorption by inhibiting osteoclast recruitment and adhesion and also decreasing their lifespan and activity (47). Denosumab is an antibody specific for RANKL, which binds to RANKL to prevent its interaction with its receptor RANK. This leads to decreased osteoclast differentiation and activity and thereby leading to decreased bone resorption (48). Based on clinical trials, Denosumab administered subcutaneously twice a

year for 36 months significantly decreased the risk of fractures and also increased BMD compared to alendronate (49,50). Multiple side effects were recorded during clinical trials for Denosumab and bisphosphonates including nasopharyngitis, back pain, bronchitis, arthralgia, constipation and pain in extremities (51). McCombs *et al.*, using data from a large health insurer discovered that the one-year compliance rates for osteoporosis therapies, bisphosphanates, raloxifene, and hormone replacement therapies, were less than 25% (55). Another study conducted by Yood *et al.*, interviewed 176 patients just starting any sort of therapy, and found a compliance rate of 70.7% with bisphosphonates therapy and 69.3% with estrogen therapy after one year or later (56).

Raloxifene acts as a selective estrogen receptor modulator and is able to increase BMD without increasing risk of breast cancer, like estrogen might possibly do (52). However it also has undesirable side effects such as thromboembolic events and neuro-emotional events such as nervousness, insomnia and emotional instability (53). Teriparatide is a recombinant parathyroid hormone fragment that has been shown through phase III clinical trial to reduce fracture rates in elderly women who have already experienced a fracture, and is generally used as a last resort for patients with severe osteoporosis because of the high cost and side effects such as leg cramps and dizziness (46, 54).

Because of the increase population at risk, specifically post-menopausal women, the great economic burden, and the side effects and decreased compliance rates of prescription drugs, it is necessary to place an importance in research for prevention and treatment of the disease to decrease risks and health care costs (57).

Role of Estrogen in Development of Postmenopausal Osteoporosis

Estrogen is a hormone that is widely known for its role in the female reproductive system and sexual characteristics but it is also important in males for spermatogenesis and fertility (58).

Estrogen is also vital in maintaining bone homeostasis in both genders, and when removed in women post menopause, sometimes results in osteoporosis. Estrogen receptors are found on both cortical and trabecular bone, however trabecular bone tends to have a higher expression of estrogen receptor-β , and cortical bone tends to express more estrogen receptor-α. It has been well documented that estrogen withdrawal due to menopause causes a decrease in bone mass (59). After menopause, the amount of osteoclasts in trabecular bone increases and the rate at which women experience bone loss increases by 10-fold (34).

Estrogen withdrawal affects bone resorption and formation through many different mechanisms. Estrogen deficiency has been linked to increased bone resorption by increased osteoclast formation, recruitment, and lifespan (by reducing apoptosis) (9). Estrogen deficiency is also responsible for decreased bone formation through increased osteoblast apoptosis and by limiting the activity of mature osteoblasts (9). All of these actions together increase the activation and frequency of basic multicellular units (BMU), the anatomical space which harbor the activities of osteoblasts and osteoclasts combined, increase remodeling space, cortical porosity, resorption area of trabecular bone, and erosion depth (59).

The mechanism of action through which estrogen withdrawal exhibits its effects is mainly through increased inflammation and oxidative stress (9). Estrogen deficiency increases inflammation is through promotion and inhibition of inflammatory cytokine genes, specifically TNF-α, IL-6, and M-CSF. When the estrogen receptor becomes activated it is able to bind to NF-κB and prevent the transcription of IL-6 genes. Similarly, activated estrogen receptor decreases JNK activity, which leads to decrease in activator protein 1, reducing the production of TNF-α genes (60). The withdrawal of estrogen would then lead to an uncontrolled production of these cytokines. Another main effect of estrogen withdrawal is the increase in TNF producing T-cells (61,62). Under normal conditions, estrogen balances the production of IL-7 by increasing TGF-β gene expression. Without this effect of estrogen, there is an increase in IL-7 production, which

stimulates TNF producing T-cell activation and expansion from the thymus and travels to the bone marrow. Effects of estrogen deficiency on IL-7 production is also seen directly in the bone marrow, where it increases hematopoietic stem cells turn into lymphoid progenitors, which will consequently increase thymic output of T-cells (9,61). Estrogen deficiency causes bone loss by increased inflammation which leads to increased osteoclast- mediated bone resorption by direct effects on osteoclasts, and indirect effects by activating RANKL and decreasing OPG production (63,64).

ROS have also been implicated in the development and progression of postmenopausal osteoporosis (65). Another important role for estrogen in the body is that it exhibits antioxidant activity by increasing the expression of glutathione peroxidase in osteoclasts. The increase production of glutathione peroxidase neutralizes hydrogen peroxide produced by osteoclasts, which is needed in the bone resorption process (66). Along with this the production of ROS decreases osteoblast lifespan and disrupts the process of bone formation, by decreasing differentiations of progenitor cells (36). Together inflammation and oxidative stress create an environment that feeds bone resorption mechanisms and blocks bone formation. The following sections take in depths look at the mechanisms behind the harmful effects of inflammation and oxidative stress on bones.

Role of Inflammation

A relationship between age-related inflammation and the development of osteoporosis also exists (67). Chronic secretion of inflammatory cytokines take part in the development and progression of postmenopausal osteoporosis. In addition to RANKL, specific pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α have been shown to take part in the bone remodeling process as well as in the development of postmenopausal osteoporosis through the withdrawal of estrogen. Age, as well as impaired immune function contribute to the release of pro-inflammatory cytokines and thus to the progression of osteoporosis (67).

As discussed earlier, estrogen deficiency leads to increased production of TNF-α by up-regulating gene expression through reducing the corepressor complexes that limit gene expression, and also through increasing activated TNF producing T-cells. TNF-α is the most effective inflammatory cytokine that increases osteoclasts differentiation and activation. Ritchlin *et al.* (68) demonstrated that mice overexpressing TNF-α, or mice injected with TNF-α showed an increase in osteoclast precursors. TNF-α also decreases osteoblast activity and increase RANKL secretion by macrophages through stimulating gene expression (15). TNF-α participates in intracellular mechanisms closely related to RANKL to activate NF- κ B and therefore increase osteoclastogenesis. TNF-α interacts with two different cell receptors on the preosteoclasts and osteoclasts: TNF-α receptor one (TNFRI) and TNF-α receptor two (TNFRII). These two receptors have different functions. When a soluble form of TNF-α interacts with TNFRI it recruits proteins that induce apoptosis. At the same time, TNFRI is also involved in the pathway that activates NF-κB, which produced an anti-apoptosis effect. Membrane bound TNF-α interacting with TNFRII acts only through intracellular mechanisms that activate NF-κB and promote osteoclastogenesis (69).

Estrogen deficiency also affects the production of other inflammatory cytokines such as IL-1 and IL-6 resulting to increased bone loss. IL-1 and IL-6 are two other powerful inflammatory cytokines that can promote bone loss and are also induced by estrogen withdrawal. IL-1 takes part in osteoclastogenesis by the activation of NF-κB and is also released in response to TNF-α (69). IL-1 has also been shown to increase RANK-L mRNA steady state levels in osteoblasts, therefore stimulating osteoclastogenesis (70). IL-6 on the other hand, secreted from osteoblasts and stromal cells, works to increase bone resorption by promoting osteoclast differentiation and activation. Most of the inflammatory effects of IL-6 occur through its effects in increasing other bone resorbing inflammatory cytokines like TNF-α and IL-1 (69). All of these

increases in inflammatory cytokines leave an imbalance between osteoblast and osteoclasts activity, while promoting oxidative stress also.

Role of Oxidative Stress

Oxidative stress, which is due to accumulation of ROS, is linked to osteoporosis (36) ROS can be produced in numerous locations in the cell including cytoplasm, endoplasmic reticulum, mitochondria and cell membrane (36). Once ROS are produced, they start a cycle of cell destruction affecting the lipid membranes, proteins and DNA, which can contribute to osteoblast apoptosis (36,71). Along with direct effects of ROS on cells, they disrupt signaling in bone coupling that occurs during the bone remodeling process by decreasing bone formation and increasing RANK-L production (36).

The decreased bone formation rate and bone mineral density (BMD) in osteoporosis has been linked to increased levels of pro-oxidants or decreased levels of anti-oxidants in the body (72,73,74). Estrogen acts like an antioxidant for osteoclasts by mediating the production of the glutathione peroxidase. The removal of estrogen not only diminishes a molecule in the body with antioxidant effect but also stimulates the body to produce reactive oxygen species (ROS) specifically hydrogen peroxide and superoxide anion (66). ROS that are not reduced by molecules with antioxidant effects such as estrogen increase lipid accumulation, which in turn increases osteoblasts apoptosis (36). The process of programmed cell death produces its own set of ROS. Along with estrogen's role in producing ROS, inflammatory cytokines can also stimulate their production (75,76,77).

Studies have suggested that osteoclasts use hydrogen peroxide to facilitate in bone resorption (77,78). Glutathione peroxidase, which is responsible for the degradation of hydrogen peroxide, is found in large amounts in osteoclasts, possibly as a protective mechanism for the cell, and for regulation of bone resorption. Estrogen as mentioned earlier, is one of the factors that

mediate the expression of glutathione peroxidase in osteoclasts (66). The increased amount of ROS from estrogen withdrawal increases TNF-α activity, which in turn activates NF- κ B for osteoclastogenesis. Osteoclast formation through the RANK-L/RANK system requires ROS production to function, and similarly RANK-L production in osteoblasts is increased in response to ROS (36,79,80).

ROS can also contribute to osteoporosis through the disruption of bone formation by diminishing differentiation of progenitor cells into osteoblast and once formed, by decreasing osteoblasts lifespan (36). Runx2, an important transcription factor, and alkaline phosphate (ALP), a protein required for mineralization of bone, colony-forming units osteoblast (CFU-O), and type 1 collagen are some of the key components involved in the osteoblasts differentiation process (36, 37,38). Bai *et al.* (37) showed that hydrogen peroxide was able to significantly reduce ALP activity, CFU-O formation, expression of type 1 collagen, and phosphorylation or activation of Runx2. Another mechanism by which ROS acts on osteoblasts is by reducing its lifespan (36). Increased ROS inside an osteoblasts will induce apoptosis, however osteoblasts generally have a protective mechanism in place through the protein glutaredoxin 5 (Grx5). Grx5 is able to reduce the ROS inside the cell mainly by reducing the action of manganese superoxide dismutase (36). Together the research on the harmful effects of ROS suggests a vital role for estrogen in maintaining bone health. Reducing inflammation and oxidative stress would be good preventative strategy for women inflicted with postmenopausal osteoporosis.

Inflammation and ROS are clearly implicated in the development and progression of postmenopausal osteoporosis and provide direction for prevention and treatment therapies. Fruits and vegetables have many bioactive components that have antioxidant and anti-inflammatory properties and therefore may be beneficial in bone health (20). This thesis will focus on how watermelon because of its bioactive components discussed in detail below, could possibly be a beneficial fruit for postmenopausal women.

Watermelon

Watermelon

In 2010, watermelon (*Citrullus lanatus*) was produced at 4.1 billion pounds in the United States and a report from 2008, states American's ate 15.4 pounds per capita of fresh watermelon (81). Watermelon is an important crop in the United States as it bought in $492 million dollars in sales in 2010 (81). The commonly ingested portion of watermelon is the flesh, however the rind contains nutrients as well. In one cup of fresh watermelon flesh (no rind) there is 46 calories with about 0.2 grams of fat, 0.9 grams of protein, and 11.5 grams of carbohydrates. It has about 0.61 grams of dietary fiber and 139 grams of water, which is approximately 92% by weight. Watermelon contains important nutrients that can affect bone health including lycopene, β-carotene, vitamin C, cucurbitacin E, and an amino acid citrulline (82,83).

Lycopene

Lycopene is one of the most potent antioxidants in the human body that has a high singlet oxygen (O_2) quenching ability, two times higher than β-carotene and ten times higher than α-tocopherol (84). Animals including humans are unable to synthesize lycopene but plants and microorganisms can, therefore we obtain it from our diet. The lycopene content of watermelon has been reported to be between 23.0 to 72.0 μg/g wet weight (85). In plants, lycopene absorbs light from the sun to protect the plant from photosensitization. It belongs to the family of carotenoids whose basic structure is a polyisoprenoid with a long chain of double bonds in the center of the molecule. There is almost a perfect symmetry at the center of a carotenoid, around the central double bond (86). Unlike other carotenoids, lycopene is not a vitamin A precursor because it does not have a β-ionic ring structure at the ends of the hydrocarbon chain (85). It is responsible for the red color of the flesh of the fruit, therefore, a more ripened fruit and more colorful varieties of watermelon, have high lycopene content (87).

Lycopene is a lipid soluble compound and so it is incorporated in fat micelles and transported across the intestinal membrane through passive diffusion. Lycopene continues to follow the fat digestion process by being packaged into chylomicrons in the enterocytes and then transported through the lymph system. Lycopene was found to be concentrated in VLDL and LDL and not in HDL. It is transported into tissues that express lycopene receptors including testes, adrenal gland, and liver (85).

Previous studies have shown lycopene to be beneficial for decreasing the effects of osteoporosis mainly through its antioxidant effects (27,84). However, lycopene has other non-oxidative functions that may be important in bone health, which include inhibiting insulin-like growth factor-I (IGF-I) signaling and IL-6 expression, increasing oxidative defense, and improving the immune response (85). Mackinnon *et al.* (24) demonstrated that women who took lycopene supplements had much lower serum oxidative stress markers and bone resorption markers. Studies have shown that increased intake in elderly men and women of carotenoids and lycopene decreased risk of hip fracture and also increased bone mineral density (27,88). Rafi *et al.* (89) demonstrated that lycopene is also involved in decreasing inflammation by mediating nitric oxide production, through regulating nitric oxide synthase, in LPS-induced mouse macrophages.

β-carotene

In addition to lycopene, another carotenoid found in watermelon is β-carotene. The content in red watermelon is greater than or equal to 32.2 μg/100 g wet weight and is considered a fruit high in β-carotene (90). β-carotene has a similar structure to lycopene however it has a β-ionone ring structure at each end, which allows it to be a precursor for retinol (85). β-carotene needs to be enzymatically digested before it is absorbed because it forms a complex with proteins in food sources. Because it is lipid soluble, it is incorporated into fat micelles for absorption by passive diffusion or possibly by a carrier protein scavenger receptor class B type 1 (SR-B1) (91).

Within the enterocyte β-carotene is converted into retinol by β-carotene 9' 10' dioxygenase or by β-carotene 15,15' –mono-oxygenase (92). Not all β-carotene is converted into retinol and the amount that is converted has an efficiency rate of 50% (12 μg of β-carotene is equivalent to 1 μg of retinol). The amount of β-carotene converted into retinol in the liver depends on the body's physiological need and the rest of the β-carotene is delivered throughout the body via chylomicrons for storage and utilization in other tissues (91). Whatever is not taken up by tissues returns to the liver with the chylomicron remnant, and the liver can incorporate it back into VLDL for circulation or convert it into vitamin A (91).

Vitamin A produced from β-carotene has a variety of functions in vision, cell growth and differentiation, and gene expression (91). However there is also some data that suggests that Vitamin A may play a role in bone health because excess intake was related to decreased bone mineral density and deficiency was related to increased mineral deposition of bone (91). β-carotene on the other hand may help with bone health by functioning as an antioxidant (91,92). Once inside the target tissue, it can be incorporated into the inner surface of the cell membranes and has the ability to quench oxygen singlet molecules and neutralize free radicals. β-carotene is thought to work in conjunction with vitamin E, which functions primarily on the outer surface of the membrane. β-carotene has the ability to neutralize singlet oxygen molecules, created by membrane lipid peroxidation, and also specially interacts with peroxyl radicals (91, 92).

Along with the studies that demonstrated the positive antioxidant effect of β-carotene on bone health (27, 88), Helden *et al.* demonstrated that a β-carotene-rich diet suppressed inflammation in 15,15′-monooxygenase 1 knockout ($Bcmo1^{-/-}$) animal model (93). This suggests a possible role of β-carotene and vitamin A as anti-inflammatory agents as well as antioxidants in preventing bone loss caused by estrogen deficiency.

Vitamin C

Vitamin C (ascorbic acid) is another potent antioxidant found in watermelon. The vitamin C content of watermelon has been reported to be about 12.3 mg per cup (152.0 g) of fresh fruit (83). Although this is only about 20% of the daily value, 1 cup of watermelon only contains about 46 calories making each calorie rich with vitamin C (82). Humans cannot synthesize vitamin C so it must be consumed in the diet. Vitamin C is a water-soluble molecule that can be absorbed by the enterocytes through the sodium dependent vitamin C transporters 1 and 2 (SVCT 1 and 2) or if it is in the oxidized form of dehydroascorbate, through GLUT 1. It is then transported in the blood in its free form to tissues that require it (91).

Vitamin C has a variety of functions in the body including carnitine synthesis, tyrosine synthesis and catabolism, microsomal metabolism, and neurotransmitter synthesis (91). A very important function of vitamin C that is required for bone formation is collagen synthesis. Collagen is synthesized through hydroxylation reactions using prolyl and lysyl hydroxylase, enzymes that require the reduced form of iron to function and vitamin C is necessary to reduce iron back into its ferrous state (91). Ascorbic acid has been linked to increased rates of both procollagen hydroxylation and secretion from osteoblasts (94). Because it is water soluble, it can function in aqueous solution making it distinct from lycopene and β-carotene. In the ascorbic acid form, it has the potential to donate hydrogen to neutralize free radicals and interact with other important antioxidants such as vitamin E and gluthathione (91). Ascorbate perioxidase is an enzyme that converts hydrogen peroxide into water and oxygen while simultaneously creating oxidized vitamin C. Glutathione is important in reducing dehydroascorbate back into its functional ascorbic acid antioxidant form (91, 94).

Several studies have suggested that vitamin C may potentially play role in bone health and a deficiency could possibly be a risk factor for osteoporosis (95, 96). A study showed direct

effects of vitamin C on osteoblast differentiation through the stimulation of nuclear factor-E2-related factor-1 on bone marrow stromal cells (97). An *in vivo* study conducted on a mouse model by Gabbay *et al.*, demonstrated that a knock out of the genes that produce vitamin C in mice caused severe osteopenia (98). A human study conducted by Leveille *et al.*, on postmenopausal women found that increase vitamin C from dietary intake was not related to increased hip bone mineral density. However, vitamin C supplementation (113 mg/day) in women aged 55-64 years, that had never used HRT, did have beneficial effects on their hipbone mineral density (99). A combination of cell, animal and human studies all suggest a positive role for vitamin C in increasing bone health (95, 96).

Other Potential Bioactive Compounds

Cucurbitacin E, a phytonutrient belonging to the triterpenoid family, is another anti-inflammatory and antioxidant molecule found in watermelon (100). Their biological functions stems from their highly oxygenated tetracyclic structure. There are no studies to date that show a relationship of this phytonutrient to bone health, however a study done by Tannin-Spitz *et al.*, showed that cucurbitacin E had a dose-dependent reduction capacity and inhibition of oxidation in cells (100). Watermelon also contains the unique amino acid citrulline that serves as a precursor for arginine, an amino acid that is required by macrophages during an inflammatory response (28). Arginine is able to control endothelial function, leukocyte activation, innate immunity, and extracellular matrix remodeling. During an inflammatory response, arginine is quickly used up and becomes conditionally essential. Because of this conversion, citrulline may be beneficial as an anti-inflammatory agent (28). Studies have also shown that citrulline is able to increase production of nitric oxide in the body therefore being beneficial for vasodilation and cardiac health (101). The antioxidant and anti-inflammatory functions of this nutrient could possibly be beneficial in postmenopausal osteoporosis, although no direct connections have been made to date.

All of these bioactive components from watermelon combined, lycopene, β-carotene, vitamin C, Cucurbitacin E and citrulline, can potentially exert anti-inflammatory and antioxidant effects in postmenopausal women. This thesis using varying doses of watermelon looks at the extent to which it may be able to provide beneficial effects for skeletal health. Additionally, we will explore other parameters that watermelon could possibly improve like glucose and lipids in ovariectomized mice.

CHAPTER III

METHODOLOGY

Diet Formulation and Preparation

The dietary treatments for this study were control (AIN-93M), 1% (w/w) watermelon (WM), 10% (w/w) WM, and 25% (w/w) WM. Watermelon was obtained from a local supermarket, the rind was removed, and the remaining pulp was freeze-dried. The freeze-dried watermelon pulp was incorporated into the AIN-93M diet formulation (102). All diets were isocaloric, isonitrogenous and had the same calcium, phosphorus and fiber content **(Table 1)** and prepared at Harlan Laboratories (Madison, WI). Macronutrient, energy as well as calcium and phosphorus content of the diets were analyzed at N.P Analytical Laboratories (St. Louis, MO, **Table 2**).

Animal Care and Necropsy

For this study, 8-week-old C57BL/6 female mice were purchased from Charles River Laboratories (Chicago, IL). The mice were housed in plastic cages and kept on a 12 hour light: dark cycle (7 AM- 7 PM) in a temperature and humidity controlled room and allowed to acclimate into this environment for one week. During the acclimation period, mice were given *ad libitum* access to the AIN-93M control diet and deionized distilled water. After the acclimation period, mice were weighed and either ovariectomized (OVX) or sham-operated (Sham), and

randomly assigned to one of six treatment groups (n=9-13/group): sham-control, OVX-control, OVX-1% WM, OVX-10% WM, OVX-25% WM, or OVX-control-alendronate (ALN)

Mice in the OVX-control-ALN group were given alendronate injection (100 μg/kg body weight) once a week. Animals were group housed (3-5 mice/cage) and fed their respective diets for twelve weeks. Food intake was measured every three days and body weights were monitored weekly. Feces were collected on the 11^{th} and 12^{th} week of the treatment for lipid analyses. Guidelines for the ethical care and treatment of animals from the Animal Care and Use Committee at Oklahoma State University were strictly followed.

After 12 weeks of dietary treatment, the mice were sacrificed and specific tissues were harvested. The mice were anesthetized with ketamine/xylazine cocktail (100.0/10.0 mg/kg body weight) and body composition was assessed by GE Lunar Piximus with Series Software version 1.4x (GE Medical Systems, LunarPIXI, Madison, WI). Blood samples were obtained from the carotid artery and placed into ethylenediaminetetraacetic acid (EDTA) coated tubes. Plasma was separated by centrifugation at 1500 x *g* for 20 min at 4°C. Aliquots of plasma were frozen and kept at -80°C for later analyses of lipids, glucose, antioxidants, and bone and anti-inflammatory markers. The thymus, a fat pad of visceral adipose tissue, uterus, spleen and liver were collected, weighed, and snap-frozen in liquid nitrogen. The intestines were removed and flushed with physiological saline for collection of cecal content.

Bone Collection and Analyses

The spine, tibia, and femur were collected and cleaned of adhering tissue for DXA and micro-computed tomography (μCT) analyses. Bone mineral density (BMD), area (BMA), and content (BMC) of the tibia and vertebrae were assessed with GE Lunar Piximus with Series Software version 1.4x (GE Medical Systems, LunarPIXI, Madison, WI). (Fitchburg, WI).

The microarchitectural parameters of the tibia and vertebra were assessed using μCT (MicroCT40, SCANCO Medical, Switzerland). The proximal tibial metaphysis and mid-diaphysis were used to analyze trabecular and cortical bone, respectively. Scans of the tibial metaphysis were performed at a resolution of 2048 x 2048 pixels, (i.e. 6 μm each slice). Semi-automated contours were placed beginning at 60 μm (10 slices) distal to the proximal growth plate and the volume of interest (VOI) included 882 μm (147 slices). The acquired images were analyzed at a threshold of 325 and a sigma and support of 1.2 and 2.0, respectively. Trabecular parameters evaluated included bone volume (BV), trabecular volume (TV), bone volume expressed per unit of total volume (BV/TV), trabecular number (TbN), trabecular thickness (TbTh), trabecular separation (TbSp) connectivity density (ConnDens) and structural model index (SMI). Analysis of cortical bone was evaluated by analyzing a 52 slice VOI at the mid-point of the tibia. Assessment of cortical bone included cortical porosity, thickness, area, and medullary area of the tibial mid-diaphysis. The acquired images were analyzed at a threshold of 260 and a sigma and support of 0.8 and 1.0, respectively.

Analyses of the spine were performed by acquiring images at a resolution of 1024 x 1024 pixels, 80μm from the dorsal and caudal growth plates. Similar to the tibial analysis, semi-automated contours were placed to assess secondary spongiosa within the VOI. The images were analyzed at a threshold of 325, and a sigma and support of 1.2 and 2.0, respectively.

Liver RNA Extraction and Quantitative Real-Time PCR

RNA Extraction

Whole liver (n=6/group) was pulverized using liquid nitrogen and approximately 200 mg of liver was weighed out. The liver tissue was homogenized in nuclease-free tubes, using VWR PowerMAX mechanical homogenizer (VWR International, Radnor, PA) in RNA STAT60 (1 mL/50 mg of tissue) (TEL-TEST, Inc., Friendswood, TX). Homogenate was then stored at room

temperature for 5 minutes to allow complete dissociation of nucleoprotein complexes and then 200 μL of ACS grade chloroform ($CHCl_3$) was added per 1 mL of RNA STAT60. The samples were vigorously shaken for about 15 seconds and then allowed to incubate at room temperature for about 3 minutes before centrifugation at 3,500 rpm (12,000 x *g*) at 4°C for 15 minutes. The upper aqueous layer was transferred to a new nuclease-free tube and ice-cold 0.5 mL ACS grade isopropanol was added for every 1 mL of RNA STAT60 used. The samples were then vortexed and stored at -80°C overnight to precipitate the RNA. Samples were thawed on ice the following day and centrifuged at 10,500 rpm for 10 minutes at 4°C. The supernatant was discarded and RNA pellet was washed with ACS grade 70% ethanol and centrifuged at 7,500 x *g* for 5 minutes at 4°C. The ethanol was discarded and the pellet was allowed to air dry for 5-10 minutes. The RNA pellet was re-suspended using diethylprocarbonate treated water (DEPC H_2O) (0.5 mL for every 200 mg of tissue). A Nanodrop Spectrophotometer (Rockland, DE) was used to determine RNA concentration and purity (using the ratio of absorbance at 260 and 280 nm, A_{260}/A_{280}).

cDNA Synthesis

RNase free DNase I (Roche Diagnostics, Indianapolis, IN) was diluted to 1/5 the original concentration using DEPC H_2O. DNase reaction was set up in an 8-strip PCR tubes with flat caps (Axygen, INC., Union City, CA) on ice. Each reaction consisted of 0.32 μL DNase I (1/5 original concentration), 2 μg RNA, 3.36 μL of 25 mM $MgCl_2$ (Roche Diagnostics, Indianapolis, IN), and DEPC H_2O to a final volume of 20 μL. The samples were then placed in a TGradient Thermocycler (Biometra, Goettingen, Germany) set at 37°C for 30 minutes, 75°C for 10 minutes, and a 4°C soak cycle.

A mastermix consisting of 1μL Superscript II RTase (Invitrogen by Life Technologies, Carlsbad, CA), 20 μL 5X PCR buffer (Invitrogen by Life Technologies, Carlsbad, CA), 10 μL 0.1M DTT (Invitrogen by Life Technologies, Carlsbad, CA), 20 μL 10mM dNTP mix (Roche

Diagnostics, Indianapolis, IN), 10 μL 0.8 mg/mL random hexamers (Roche Diagnostics, Indianapolis, IN), and 19 μL of DEPC H_2O, was added to each sample for a total volume of 100 μL. The samples were then placed once more in a TGradient Thermocycler set at 25°C for 10 minutes, 42°C for 50 minutes, and 72°C for 10 minutes, and then a 4°C soak cycle, until samples were removed. The cDNA was stored at -20°C until further analysis.

Real-Time PCR

Quantitative real time PCR was used to determine the presence and quantity of catalase (CAT), glutathione peroxidase 3 (GPX-3), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF- α). Primers were prepared by combining 6.25 μL of the forward primer, 6.25 μL of the reverse primer, and 487.5 μL of DEPC H_2O for a total concentration of 0.3 μM primer mix. 5.0 μL of cDNA was analyzed using Power SYBR green chemistry (Applied Biosystems, Foster City, CA) on the Applied Biosystems 7300 Real Time PCR (Foster City, CA). All results were calculated by the comparative cycle number at threshold (C_T) method, using cyclooxygenase (Cyclo) as the invariant control. The primers used for qPCR are shown in **Table 3**.

Plasma Analyses

The BioLis 24i clinical chemistry analyzer from Carolina Liquid Chemistries Corporation (Brea, CA) was used to determine plasma concentrations of glucose, non-esterified fatty acids (NEFA), total cholesterol, and triglycerides. Kits were purchased from Carolina Liquid Chemistries Corporation (Brea, CA) except for NEFA which was purchased from Wako Diagnostics (Richmond, VA) and the manufacturer's instructions were strictly followed.

The reaction of glucose, with the enzymes hexokinase and glucose-6-phosphate dehydrogenase produces NADH whose absorbance was measured at 340 nm after. NEFA is measured by a purple color produced by the oxidative condensation of 3-methyl-N-ethyl-N(β-hydroxyethyl)-aniline (MEFA) and 4 aminoantipyrine. The reaction occurs in the presence of

added peroxidase and hydrogen peroxide that is produced as a byproduct of acyl-CoA and added acyl-CoA oxidase. Acyl-CoA is only produced in the presence of fatty acids plus added acyl-CoA synthetase, so therefore the color measured at 550 nm is directly proportional to the amount of NEFA present in the plasma.

Cholesterol is measured by the addition of cholesterol esterase to the plasma sample to convert all cholesterol esters into free cholesterol and free fatty acids. Cholesterol then in the presence of oxygen and cholesterol oxidase forms cholest-4-ene-3-one and hydrogen peroxide. With the addition of peroxidase the hydrogen peroxide aids in the oxidative condensation of 4-aminoantipyrine and p-hydroxybenzoate into quinoneimine, which can be measured at 546 nm. Triglycerides were measured using a technique that breaks it down to glycerol and fatty acids using microbial lipoprotein lipase. The glycerol then with the help of added ATP, glucokinase, and magnesium, is converted into glycerol-3-phosphate and ADP. In the presence of oxygen and glycerol-3-phosphate oxidase, the glycerol-3-phosphate is converted to dihydroxyacetone phosphate and hydrogen peroxide. The hydrogen peroxide aids in the oxidative condensation of 4-aminoantipyrine and HDCBS (3,5-dichloro-2-hydroxy-benzenesulfonicacidisodiumsalt) that produces a color that can be measured at 500 nm and is proportional to the amount of triglycerides in the plasma.

Plasma catalase and glutathione peroxidase activity were determined using commercially available kit from Cayman Chemical Company (Ann Arbor, MI) and results were read using the Synergy HT spectrophotometer (BioTek Instruments Inc., Winooski, VT). The catalase kit utilizes the peroxidatic function of catalase to determine the amount present in the sample. With the addition of methanol and hydrogen peroxide to the sample, the catalase produces formaldehyde. Formaldehyde reacts with the Purplad (4-amino-3-hydrazino-5-mercapto-1,2,4-trizole) to turn a purple color and the absorbance was read at 540 nm. Glutathione peroxidase (GPX) activity is measured indirectly by a coupled reaction with glutathione reductase.

Hydroperoxide and reduced glutathione (GSH) produce oxidized glutathione (GSSG) in the presence of GPX. It is then recycled by glutathione reductase, which is accompanied by the oxidation of NADPH to NADP+ that reduces absorbance at 340 nm. The reduction in A_{340} is proportional to the amount of GPX present in the sample.

Liver and Fecal Lipids

Lipid extraction

Feces and frozen liver tissue were ground, weighed into a filter paper and soaked in 25 mL of a 2:1 solution of choloform:methanol (v/v) for three days. After three days of extraction, the filter paper was removed, and 8 mL of 0.73% saline solution was added to the choloroform:methanol and vortexed. The solution was allowed to sit for a few minutes until the two layers were distinctly separated. The aqueous top layer was aspirated off and the bottom organic layer was poured into pre-weighed, oven dried aluminum pan. The solution was air-dried and dried in the oven at 100°C for about an hour. The pans were allowed to cool in a dessicator and weighed for determination of lipid weight. A 2 mL aliquot was taken from the aspirated chloroform: methanol solution of the liver for cholesterol analysis.

Determination of Liver Cholesterol

A 2 mL aliquot of the chloroform:methanol solution (from the liver lipid analysis) was transferred into a screw cap vial to determine cholesterol content of the liver. Cholesterol standards and control serum were also put into separate vials and evaporated with nitrogen gas. The aliquot of the chloroform:methanol extract of the liver samples were also dried under nitrogen gas. After standards, samples and controls were dried, a saponification solution consisting of 15% KOH, ethanol, and pyrogallic acid, was added to the tubes. The tubes were heated in a rocking water bath at 90 °C for 20 minutes, briefly cooled, and 5 mL of distilled water and 10 mL of hexane were added. The tubes were vigorously mixed and the two phases were

allowed to separate. A 5 mL aliquot of the upper hexane phase was separated into another screw cap vial and was allowed to evaporate again. Following this evaporation step, concentrated sulfuric acid, $FeSO_4 \bullet 7H_2O$ in 17.4 M (99.7%) acetic acid, and a mixture of acetone and ethanol (1:1, v/v) was added to the vials and were allowed to sit for 10 minutes to develop color. The results were read with a DU 800 Spectrophotometer (Beckman Coulter, Inc., Pasadena, CA). The concentration of cholesterol in the sample was obtained from the standard curve.

Statistical Analyses

Statistical analyses involved computation of means and standard deviation (SD) for each of the treatment groups using SAS version 9.3 (SAS Institute, Cary, NC). Analysis of variance and least square means were calculated using the general linear model procedure and the means were compared using Fisher's least significant difference for comparing groups. Differences were considered significant at $P < 0.05$.

Table 1. Diet Composition

Ingredients	AIN-93 M	1% Watermelon	10% Watermelon	25% Watermelon
	g/kg Diet			
Watermelon g/kg Diet		**10**	**100**	**250**
CARBOHYDRATE				
Total	721	726.847	721.102	711.507
Cornstarch	466	456.397	377.572	246.177
Sucrose	100	107.33	107.33	107.33
Dextrinized Cornstarch	155	155	155	155
Watermelon (81.2%)	-	8.12*	81.2*	203*
PROTEIN				
Total	140	140	140	140
Casein	140	139.14	131.39	118.48
Watermelon (8.61%)	-	0.861*	8.61*	21.525*
FAT				
Total	40	40	40	40
Soybean Oil	40	39.768	37.68	34.2
Watermelon (2.32%)	-	0.232*	2.32*	5.8*
FIBER				
Total	50	50	50	50
Cellulose	50	49.897	48.97	47.425
Watermelon (1.03%)	-	0.103*	1.03*	2.575*
VITAMIN MIX (AIN 93VX)	10	10	10	10
MINERAL MIX				
Total	35	35	35	35
Mineral Mix (Ca-P Def; TD 98057)	13.4	13.4	13.4	13.4
Calcium Carbonate ($CaCO_3$); 40.04% Ca	12.5	6.090	6.440	7.03
Calcium from watermelon (0.0914%)	-	0.00914*	0.0914*	0.2285*
Calcium Phosphate, dibasic	2.4	8.67	7.91	6.65
Phosphorus from watermelon (0.253%)	-	0.0253*	0.253*	0.6325*
Sucrose	6.7	6.840	7.250	7.920
CHOLINE BITARTRATE	2.5	2.5	2.5	2.5
L-CYSTEINE	1.8	1.8	1.8	1.8
TERT-BUTYLYL-HYDROQUINONE	0.008	0.008	0.008	0.008

*Assayed by N.P Analytical Laboratories (St. Louis, MO)

Table 2. Diet Analyses

Analyte	AIN-93 Control	AIN-93+ WM 1%	AIN-93+ WM 10%	AIN-93+ WM 25%
Moisture %	9.05	13.8	13.3	14.0
Protein %	12.5	11.7	10.2	12.2
Fat %	4.55	4.47	4.49	4.25
Fiber-Crude %	2.97	2.99	3.31	3.88
Ash %	2.31	2.07	2.19	2.84
Calcium %	0.496	0.465	0.492	0.489
Phosphorus %	0.303	0.290	0.286	0.281
Carbohydrate (by calc.) %	71.6	68.0	69.8	66.7
Calories (by calc.) kcal/100g	377	359	360	354

Diets were analyzed by N.P Analytical Laboratories (St. Louis, MO)

Table 3. List of Primer Sequences used for Real-Time PCR

Symbol	Name	Primer Sequence	Accession Number
Cyclo	Cyclophilin	For-5`tggagagcaccaagacagaca Rev-5`tgccggagtcgacaatgat	NM_011149
CAT	Catalase	For-5'ccgagtctctccatcaggttt Rev-5'tcatgtgccggtgaccat	NM_009804.2
GPX-3	Glutathione Peroxidase-3	For-5'aactcggagatactccccagtct Rev-5'gctggaaattaggcacaaagc	NM_008161
IL-6	Interleukin-6	For-5'gaggataccactcccaacagacc Rev-5'aagtgcatcatcgttgttcataca	NM_031168

The criteria used for primer design/validation is the amplicon must span an intron, template titration must have an efficiency slope of -3.3, and demonstrate the formation of a single dissociation curve.

CHAPTER IV

RESULTS

Weekly Body Weights, Food Intake, and Tissue Weights

All mice had similar body weights at the start of the dietary treatment **(Table 4)**. After only one week of treatment, OVX mice receiving the two high doses of watermelon (OVX-WM 10% and 25%) had higher body weights compared to all the other treatment groups **(Figure 1)**. By week two, all WM-fed groups had significantly higher weights than the groups fed the control diet (Sham-control, OVX-control, and OVX-ALN-Control). The body weight of the WM-fed groups continued to be significantly higher throughout the treatment period, despite having lower food and caloric intake compared to the Sham-control and OVX-control **(Table 4)**. Among the watermelon groups, the mice that were given the 25% dose had the highest body weight at the end of the 12 week dietary treatment.

As expected, uterine weights were highest for the sham group and all the other OVX groups had similar uterine weights **(Table 4)**. The sham and OVX alendronate control (OVX-ALN- control) fed groups had the lowest liver weight. There was no significant difference in liver weight between WM fed groups and the OVX-control group. Consistent with the final body weight, visceral adipose tissue weight was significantly higher in OVX-WM 25% compared to all other groups. OVX-WM 10% and WM1% also had higher visceral adipose tissue weights

compared to mice fed the control diet (OVX-control, Sham-control, and OVX-ALN-Control) but lower than the OVX-WM 25% group.

Whole Body Composition, and Tibia and Lumbar PIXImus Analysis

Percent whole body fat analyzed by x-ray absorptiometry is consistent with the weight of the isolated visceral adipose tissue **(Table 5)**. The WM fed groups had higher percent body fat and lower percent lean mass compared to the mice fed the control diet. Additionally, WM fed groups (particularly the 25% WM) had lower whole body bone mineral BMD, BMC and total area than Sham-Control and OVX-ALN-Control groups. The 10% WM group had whole body BMD, BMC and BMA that were statistically similar to the OVX-Control. The Sham-Control group had the highest BMD, BMC, and area followed by the OVX-ALN-Control group **(Table 5)**.

Isolated bone (i.e., tibia and spine) was also analyzed by x-ray absorptiometry. Tibial bone area was highest in the OVX-ALN and OVX-WM 1% groups and lowest in the OVX-25% WM group **(Table 5)**. Tibial BMC was also highest in the OVX-ALN, which is statistically similar to the Sham-Control group. Among the WM-fed groups, the OVX-WM25% group had the lowest tibial BMC while the OVX-WM1% group had the highest tibial BMC but it is statistically similar to the OVX-control group and. The pattern for tibial BMD is similar to the tibial BMC with the OVX-ALN and Sham-Control group having the highest while the WM 25% group having the lowest tibial BMD **(Table 5 and Figure 2)**.

Unlike the tibia, lumbar BMA were not statistically different among all the treatment groups (**Table 5)**. Lumbar BMC and BMD were statistically similar in the sham and OVX-ALN groups, which were also significantly higher than all the other OVX groups. There were no significant differences in lumbar BMC and BMD in all OVX-WM and OVX-control fed groups **(Figure 2, Table 5)**.

Tibial Trabecular and Cortical Bone Microarchitecture (Table 6)

Tibial trabecular bone volume over total volume (BV/TV) values were significantly different between the groups. BV/TV was highest ($p<0.05$) for OVX-ALN-control followed by the Sham-control group. All OVX-WM groups and the OVX-control group have similar BV/TV and they were lower compared to both the OVX-ALN-control and Sham-control groups.

Tibial trabecular bone connectivity density (Conn. D.) was highest for the OVX-ALN-control group and all the other OVX groups as well as the Sham-control group were similar. Structural model index (SMI) was highest for the WM 10% group which was statistically similar to all the other WM groups. SMI was lowest for the OVX ALN control group, which was similar to the Sham-control. SMI value of the OVX-1% and 25% WM as well as OVX-control groups was similar to the Sham-control group.

All the WM groups and the OVX-control have similar tibial trabecular number (Tb.N) which was lower than the Sham-control and OVX-ALN control groups. Trabecular thickness (Tb.Th) was highest in the Sham control group, which was statistically similar to OVX- control, OVX-WM 1% and 10% groups. The OVX-ALN-control group had the lowest Tb.Th value. Trabecular separation (Tb.Sp) was increased in all the WM and OVX control groups and lowest in OVX-ALN control group. The WM 1% and 10% as well as the OVX control groups have similar DA values which were lower than the WM 25%, Sham-control, and OVX-ALN control groups.

The OVX-control and all doses of WM have similar cortical area, which was lower than the Sham-control and OVX-ALN groups. There were no significant differences between groups for cortical BV/TV. There were also no significant differences observed for medullary area and percent porosity of the tibial cortical bone between the groups.

Lumbar (L4) Trabecular Bone Microarchitecture (Table 7)

The BV/TV was highest in the OVX-ALN group as expected due to treatment. Sham control group had the second highest BV/TV values. The lowest BV/TV belonged to the WM 25% groups and this was comparable to WM 1% and OVX-control groups. WM10% was significantly higher than the OVX-control group. Between the three WM groups, WM 10% had the highest BV/TV, about 58% greater than WM 25% and about 47.5% higher, although not significant, than WM 1%.

Similarly, Tb.N. and Tb.Th were highest and Tb.Sp was lowest in WM10% compared to all watermelon groups, which was most likely responsible for the increased BV/TV in WM10% group. Additionally, WM10% was the only WM group that was comparable to the Sham control group for Tb.N. and Tb.Sp, although also similar to OVX-control. WM10% was able to prevent decrease in connectivity density due to OVX and was significantly higher than WM1%, WM25% and OVX-control groups. WM 10% also had the lowest SMI between WM1%, WM25% and OVX-control although not significant. However between these groups, the SMI value for WM10% was the only one comparable to sham control. There is no statistically significant difference in DA values for the lumbar trabecular bone among all the treatment groups.

Plasma Lipids, Glucose, and Antioxidant Enzymes (Table 8)

No significant differences were observed in plasma triglycerides and non-esterified fatty acids in all groups. Plasma cholesterol concentration was highest in the OVX-WM 1% group and statistically similar to the WM 25% and OVX-control groups. The WM-10% group has plasma total cholesterol that is similar to the Sham control and OVX-ALN groups. Plasma glucose concentration was highest in the OVX-WM 25% group and lowest in the Sham-control group. The plasma glucose concentrations of the OVX-WM 1% and 10% were comparable to the OVX-ALN-control and OVX-control groups.

Catalase and GPX activity were both assessed in the plasma. There were no significant differences found in either of the antioxidant enzymes. However, GPX activity tended to be increased in the WM10% fed group, while being the lowest in the OVX-WM1% group.

Fecal and Liver Total Lipids and Liver Cholesterol (Table 9)

All OVX groups have higher liver total lipids compared to the Sham-control group. However, the WM 10% and 25%-fed groups have liver total lipds similar to the Sham-control group. There was no significant difference in liver cholesterol. Fecal total lipid was highest in OVX-WM10% group and lowest in the OVX-control group.

Liver Relative mRNA Abundance of Inflammatory Cytokines and Antioxidant Enzymes (Figures 3&4)

The mRNA abundance in the liver of the antioxidant enzymes catalase (CAT) and glutathione peroxidase 3 (GPX-3) and inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), were all quantified. The WM10% fed group had the highest liver gene expression of catalase. All the remaining groups have similar gene expression of catalase in the liver. There were no significant differences found in the liver gene expression of glutathione peroxidase as well as IL-6 and TNF-α.

Table 4. Food Intake, and Body and Tissue Weights

Parameters	Sham Control (n=12)	OVX-Control (n= 9)	OVX-WM 1% (n= 12)	OVX-WM10% (n= 13)	OVX-WM 25% (n= 13)	OVX-ALN – Control (n= 9)	P value
Food Intake							
Grams/ mouse/day)	3.5± 0.2^{a}	3.5± 0.4^{a}	3.3± 0.3^{b}	3.3± 0.3^{b,c}	3.3± 0.3^{b,c}	3.2± 0.3^{c}	<0.0001
Calories/ mouse/day)	13.0± 0.9^{a}	13.3± 1.4^{a}	11.8± 1.1^{b}	11.7± 1.1^{b}	11.8± 0.9^{b}	11.9± 1.2^{b}	<0.0001
Body Weights (g)							
Initial	22.54± 0.91	22.35± 0.94	22.67± 0.94	22.71± 0.84	22.76± 0.90	22.39± 0.63	0.8439
Final (Week 12)	23.70± 0.83^{d}	30.37± 2.86^{b}	31.61± 1.88^{a,b}	31.10± 2.67^{b}	33.41± 2.06^{a}	27.94± 2.96^{c}	<0.0001
Tissue Weights (mg)							
Uterus	72.5± 21.8^{a}	14.4± 5.3^{b}	14.2± 5.2^{b}	16.9± 9.5^{b}	14.6± 5.2^{b}	16.7± 5.0^{b}	<0.0001
Liver	954.2± 122.6^{c}	1157.8± 213.7^{a,b}	1204.2± 112.7^{a,b}	1118.5± 166.9^{b}	1272.3± 86.3^{a}	974.4± 161.1^{c}	<0.0001
Adipose tissue	394.2± 97.5^{d}	1276.7± 378.5^{c}	1675.0± 357.8^{b}	1658.5± 453.7^{b}	1993.8± 494.1^{a}	1174.4± 416.1^{c}	<0.0001

Values are mean ± SD; In a row, values that do not share the same superscript letter are significantly different ($p<0.05$)

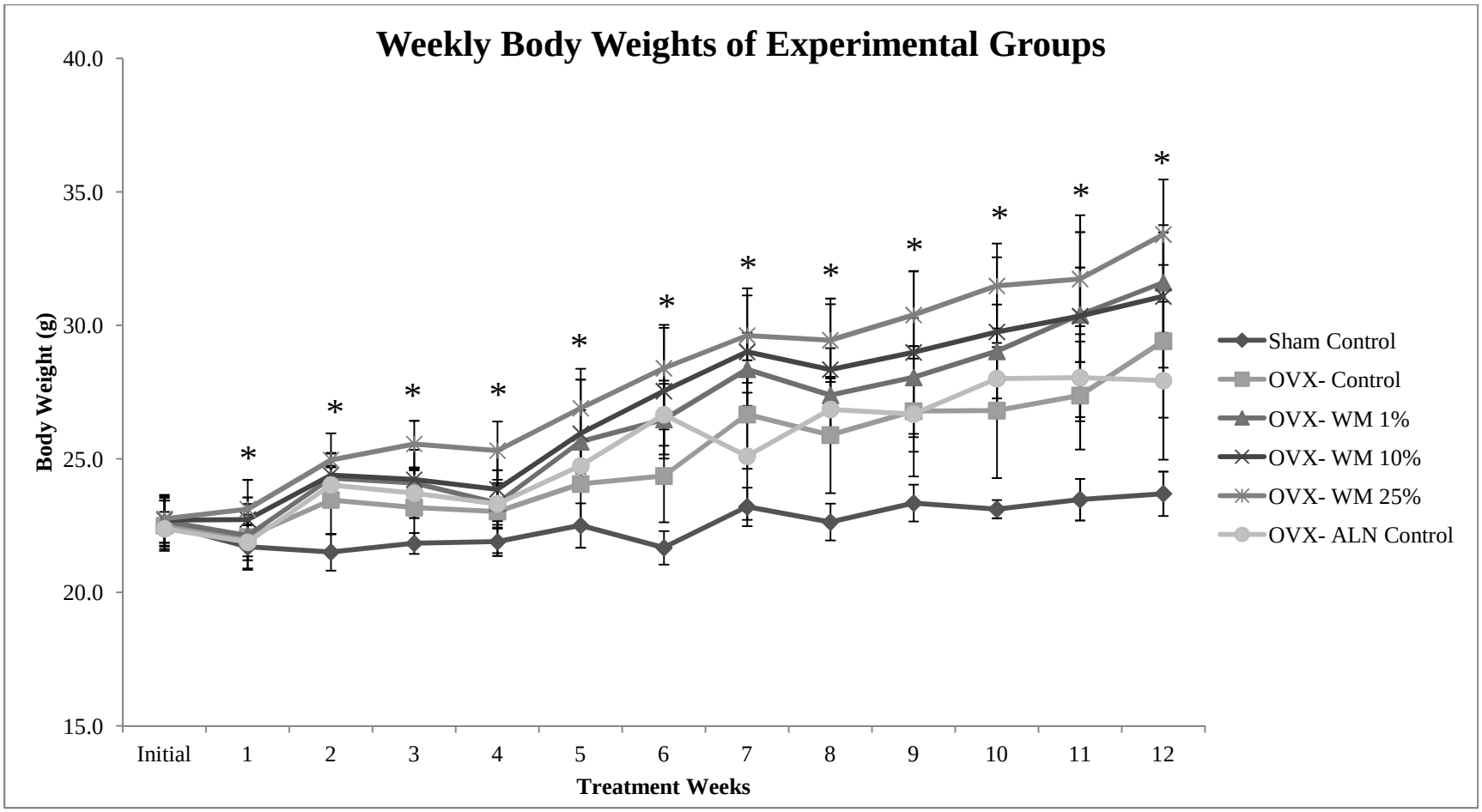

Figure 1.
Comparison of weekly body weights between groups throughout the 12-week treatment period. Asterisks denote significant difference between WM25% and OVX-control from weeks 2 through 12, and significant difference between WM10% and OVX-control from weeks 2 through 11.

Table 5. Whole Body Composition, Tibia and Lumbar Bone Parameters

Parameters	Sham Control (n=12)	OVX-Control (n= 9)	OVX-WM 1% (n= 12)	OVX-WM10% (n= 13)	OVX-WM 25% (n= 13)	OVX-ALN – Control (n= 9)	P value
Whole Body Composition							
%Fat	24.9± 1.9^{d}	33.2± 3.9^{c}	36.4± 2.5^{b}	36.7± 3.5^{b}	39.3± 2.5^{a}	33.7± 3.5^{c}	<0.0001
%Lean	75.1± 1.9^{a}	66.8± 3.9^{b}	63.6± 2.5^{c}	63.3± 3.6^{c}	60.7± 2.6^{d}	66.3± 3.4^{b}	<0.0001
BMD (mg/cm^2)	53.6± 3.4^{a}	47.1± 4.2^{b,c}	41.2± 6.3^{d,e}	43.7± 8.0^{c,d}	38.9± 6.3^{e}	49.3± 5.1^{a,b}	<0.0001
BMC (mg)	642.7± 57.9^{a}	471.6± 62.6^{b,c}	397.7± 73.5^{d,e}	423.8± 81.7^{c,d}	352.6± 59.8^{e}	516.1± 78.6^{b}	<0.0001
BMA (cm^2)	12.0± 0.8^{a}	9.9± 0.7^{b,c}	9.6± 0.5^{c}	9.7± 0.6^{c}	9.1± 0.4^{d}	10.4± 0.8^{b}	<0.0001
Tibia							
BMA (cm^2)	0.50± 0.02^{a,b,c}	0.50± 0.04^{a,b}	0.51± 0.04^{a}	0.48± 0.02^{b,c}	0.47± 0.02^{c}	0.51± 0.03^{a}	0.0110
BMC (mg)	25.55± 1.57^{a,b}	23.67± 1.66^{c,d}	24.50± 1.88^{b,c}	22.62± 1.39^{d,e}	21.62± 1.71^{e}	26.78± 1.86^{a}	<0.0001
Lumbar							
BMA (cm^2)	0.30± 0.02	0.31± 0.02	0.31± 0.03	0.30± 0.02	0.30± 0.02	0.31± 0.02	0.5700
BMD (mg/cm^2)	58.36± 3.64^{a}	46.06± 2.80^{b}	47.77± 3.66^{b}	49.03± 4.96^{b}	46.69± 2.62^{b}	58.87± 3.83^{a}	<0.0001

Values are mean ± SD; In a row, values that do not share the same superscript letter are significantly different ($p<0.05$); BMA= Bone Mineral Area; BMD= Bone Mineral Density; BMC= Bone Mineral Content

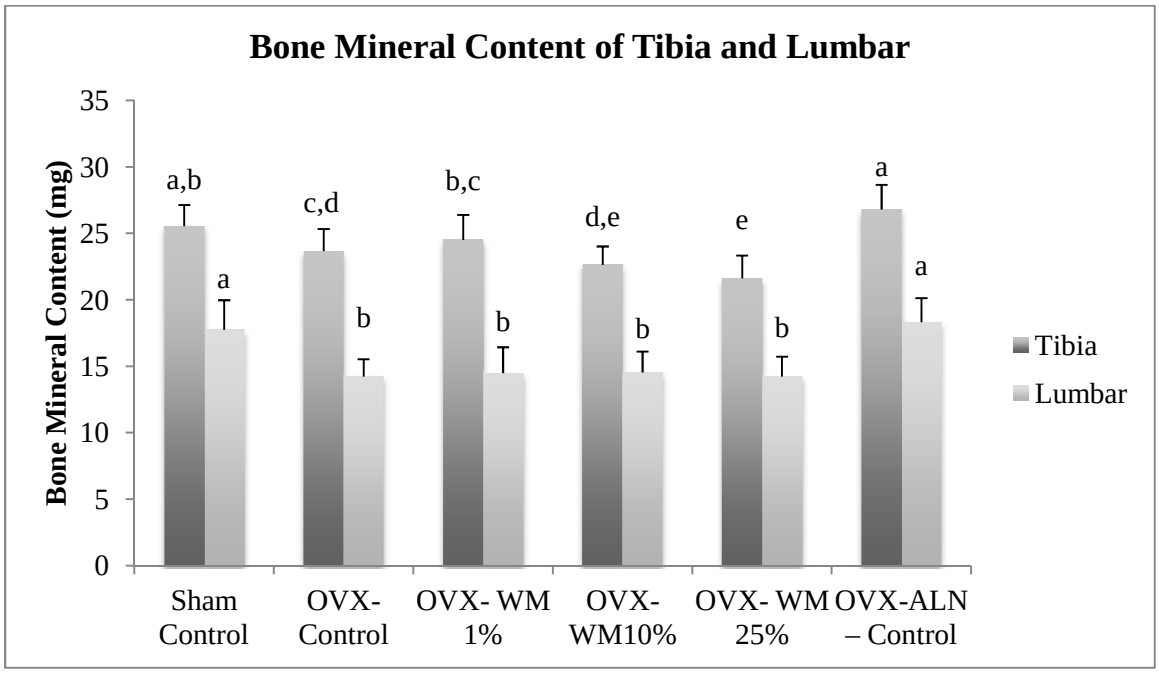

Figure 2. Comparison of bone mineral content of tibia and lumbar trabecular bone. Values are mean ± SD; Bars that do not share the same superscript letter are significantly different ($p<0.05$).

Table 6. Proximal Tibial Metaphysis and Tibial Mid-Diaphysis

	Sham Control (n=6)	**OVX-Control (n= 6)**	**OVX-WM 1% (n= 6)**	**OVX-WM10% (n= 6)**	**OVX-WM25% (n= 6)**	**OVX-ALN – Control (n= 6)**	**P value**
***Proximal Tibial Metaphysis (Trabecular Bone*)**							
BV/TV %	7.65± 0.52^{b}	5.35± 0.58^{c}	4.60± 0.74^{c}	4.85± 0.85^{c}	4.59± 0.65^{c}	9.49± 2.50^{a}	<0.0001
Tb.N [1/mm]	2.62± 0.21^{b}	2.25± 0.16^{c}	2.19± 0.14^{c}	2.24± 0.08^{c}	2.29± 0.17^{c}	3.16± 0.57^{a}	<0.0001
Tb.Th [µm]	54.98± 3.70^{a}	52.65± 5.90^{a,b}	54.80± 1.45^{a}	53.47± 2.37^{a,b}	50.02± 2.60^{b}	43.33± 2.45^{c}	<0.0001
Tb.Sp [µm]	378.78± 35.05^{b}	448.40± 46.11^{a}	462.40± 32.71^{a}	446.93± 17.28^{a}	432.17± 45.60^{a}	317.68± 60.04^{c}	<0.0001
Conn. D. [1/ mm^{3}]	37.97± 12.77^{b}	32.22± 13.36^{b}	21.30± 7.22^{b}	20.75± 11.77^{b}	19.51± 4.98^{b}	80.96± 33.23^{a}	<0.0001
SMI	2.01± 0.13^{b,c}	2.08± 0.22^{b}	2.15± 0.19^{a,b}	2.33± 0.24^{a}	2.21± 0.11^{a,b}	1.79± 0.20^{c}	0.0008
DA	1.81± 0.06^{b}	1.60± 0.22^{c}	1.61± 0.12^{c}	1.65± 0.16^{b,c}	1.84± 0.24^{b}	2.04± 0.15^{a}	0.0004
Tibial Mid-Diaphysis (Cortical Bone)							
Cortical Thickness [µm]	222.33± 9.67^{a}	196.33± 10.80^{b}	198.00± 7.13^{b}	206.17± 6.62^{b}	198.50± 11.61^{b}	220.50± 9.85^{a}	<0.0001
Cortical Area [mm^{2}]	681.30± 26.78^{a}	635.63± 46.99^{b}	618.00± 25.51^{b}	637.93± 25.98^{b}	631.25± 51.29^{b}	694.44± 31.03^{a}	0.0046
Medullary Area [mm^{2}]	10.84± 2.29	11.06± 3.22	9.13± 1.61	9.78± 1.29	9.67± 2.36	9.14± 4.18	0.7148
Cortical Porosity %	1.56± 0.31	1.71± 0.39	1.48± 0.26	1.51± 0.18	1.51± 0.29	1.41± 0.33	0.6557

Values are mean ±SD; In a row, values that do not share the same superscript letter are significantly different (p<0.05). TV= Total Volume; BV= Bone Volume; Conn.D= Connectivity Density; SMI= Structural Model Index; Tb.N.=Trabecular Number, Tb.Th.= Trabecular Thickness, Tb.Sp= Trabecular Space; DA= Degree of Anisotropy

Table 7. Lumbar (L4) Trabecular Bone Microarchitecture

	Sham Control (n=6)	**OVX-Control (n= 6)**	**OVX-WM 1% (n= 6)**	**OVX-WM10% (n= 6)**	**OVX-WM 25% (n= 6)**	**OVX-ALN – Control (n= 6)**	**P value**
BV/TV %	11.41± 1.27^{b}	5.62± 1.09^{d}	5.79± 1.35^{c,d}	8.56± 3.75^{c}	5.39± 1.46^{d}	14.25± 3.55^{a}	<0.0001
Tb.N [1/mm]	3.38± 0.16^{b}	2.60± 0.17^{c,d}	2.57± 0.36^{d}	3.03± 0.61^{b,c}	2.80± 0.21^{c,d}	3.92± 0.54^{a}	<0.0001
Tb.Th [µm]	43.32± 3.27^{a}	35.75± 2.22^{b,c}	36.38± 3.17^{b,c}	37.53± 2.58^{b}	33.48± 2.51^{c}	38.67± 2.71^{b}	<0.0001
Tb.Sp [µm]	299.62± 14.62^{c,d}	389.90± 27.71^{a,b}	399.47± 54.65^{a}	342.58± 64.08^{b,c}	363.55± 29.47^{a,b}	260.23± 36.77^{d}	<0.0001
Conn. D. [1/ mm^{3}]	133.17± 14.39^{b}	53.33± 14.75^{c}	57.74± 24.61^{c}	110.52± 80.73^{b}	56.80± 26.78^{c}	217.01± 50.66^{a}	<0.0001
SMI	1.70± 0.15^{b,c}	2.32± 0.30^{a}	2.32± 0.16^{a}	2.04± 0.55^{a,b}	2.47± 0.25^{a}	1.35± 0.57^{c}	<0.0001
DA	1.75± 0.09	1.74± 0.08	1.77± 0.05	1.69± 0.06	1.74± 0.15	1.68± 0.06	0.4153

Values are mean ±SD; In a row, values that do not share the same superscript letter are significantly different (p<0.05). TV= Total Volume; BV= Bone Volume; Conn.D= Connectivity Density; SMI= Structural Model Index; Tb.N.=Trabecular Number, Tb.Th.= Trabecular Thickness, Tb.Sp= Trabecular Space; DA= Degree of Anisotropy

Table 8. Plasma Lipids, Glucose and Antioxidant Enzymes

	Sham Control (n=12)	**OVX-Control (n= 9)**	**OVX-WM 1% (n= 12)**	**OVX-WM10% (n= 13)**	**OVX-WM 25% (n= 13)**	**OVX-ALN – Control (n= 9)**	**P value**
Lipids and Glucose							
Cholesterol mg/dL	79.3± 6.4[c]	89.6± 20.1[a,b,c]	100.1± 10.0[a]	79.9± 16.9[b,c]	92.6± 11.2[a,b]	83.4± 29.2[b,c]	0.0179
Triglycerides mg/dL	32.7± 8.8	31.7± 10.8	32.5± 8.6	32.2± 10.6	34.4± 11.2	29.4± 11.3	0.9279
Nonesterified Fatty Acids mEq/L	0.8± 0.2	1.0± 0.4	0.8± 0.2	0.9± 0.4	0.8± 0.3	0.8± 0.4	0.776
Glucose mg/dL	361.0± 55.5[c]	395.5± 85.5[b,c]	431.3± 53.5[b]	429.6± 73.7[b]	504.8± 73.01[a]	388.0± 80.9[b,c]	0.0001
Anti-oxidant Enzymes							
Catalase activity (*nm/mol/min)*	10.478± 3.582	12.531± 2.098	11.063± 3.024	10.387± 2.157	9.578 ± 3.268	11.742± 1.436	0.2351
Glutathione peroxidase activity *(nm/mol/min)*	27.470± 17.381	20.921± 29.609	13.644± 19.847	49.028± 13.732	33.817± 23.300	27.106± 27.041	0.0710

Values are mean ±SD; In a row, values that do not share the same superscript letter are significantly different ($p<0.05$).

Table 9. Fecal and Liver Total Lipids and Liver Cholesterol

	Sham Control (n=12)	**OVX-Control (n= 9)**	**OVX-WM 1% (n= 12)**	**OVX-WM10% (n= 13)**	**OVX-WM 25% (n= 13)**	**OVX-ALN – Control (n= 9)**	**P value**
Liver							
Total lipids (mg/g)	72.37± 9.94[b]	94.09± 22.1[a]	102.62± 30.51[a]	87.79± 13.11[a,b]	88.73± 18.07[a,b]	104.15± 24.46[a]	0.0062
Cholesterol (mg/g)	14.6± 13.1	12.2± 12.1	8.4± 5.8	9.6± 10.2	10.0± 6.8	13.5± 10.3	0.6328
Feces							
Total lipids (mg/g)	11.8± 0.5[b,c]	10.7± 1.6[c]	12.8± 0.9[a,b]	14.8± 1.2[a]	14.0± 1.5[a]	12.7± 0.8[a,b,c]	0.0162

Values are mean ± SD; In a row, values that do not share the same superscript letter are significantly different ($p<0.05$).

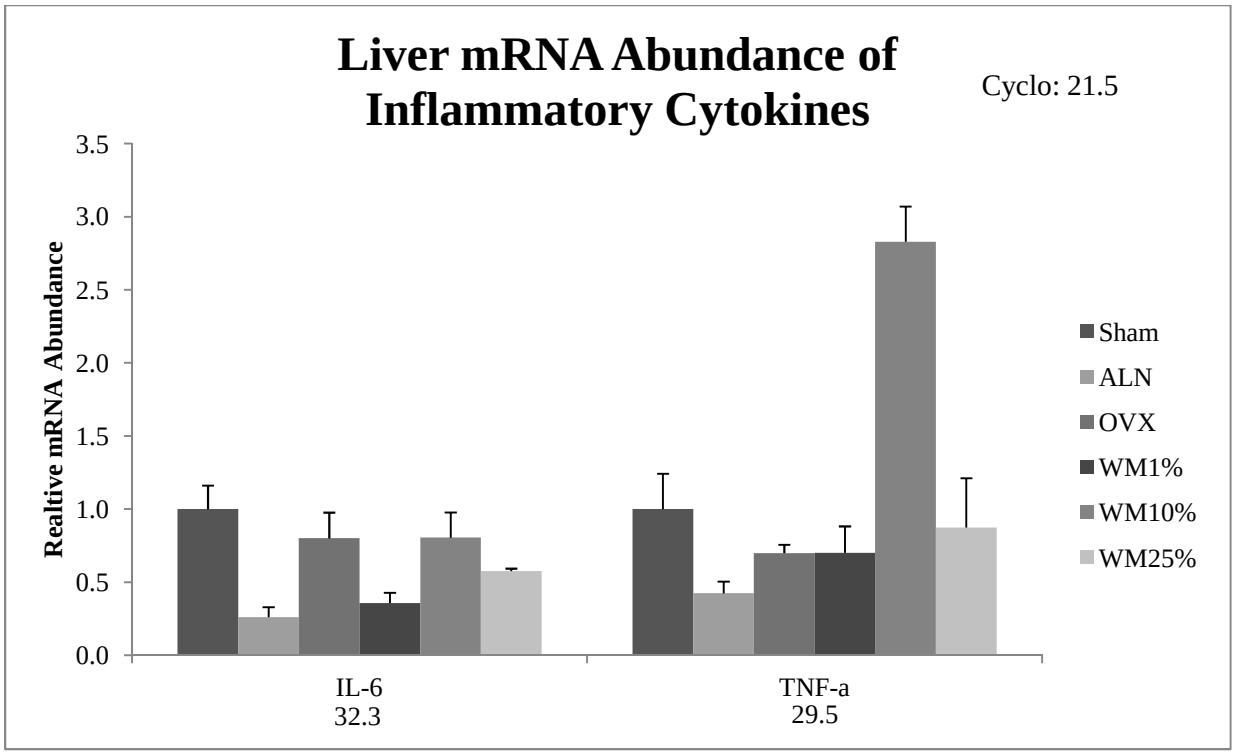

Figure 3. Comparison of liver relative mRNA abundance of inflammatory cytokines in OVX-ALN-control, OVX-Control, WM1%, WM105 and WM25% relative to Sham control group (set at 1.0). CT values for Sham control group are listed below the respective gene.Values are mean ± SEM; Bars that do not share the same superscript letter are significantly different ($p<0.05$). IL-6= interleukin 6, TNF-α= tumor necrosis factor alpha. Cyclo= Cyclophilin was used as the invariant control and had a CT value of 21.5.

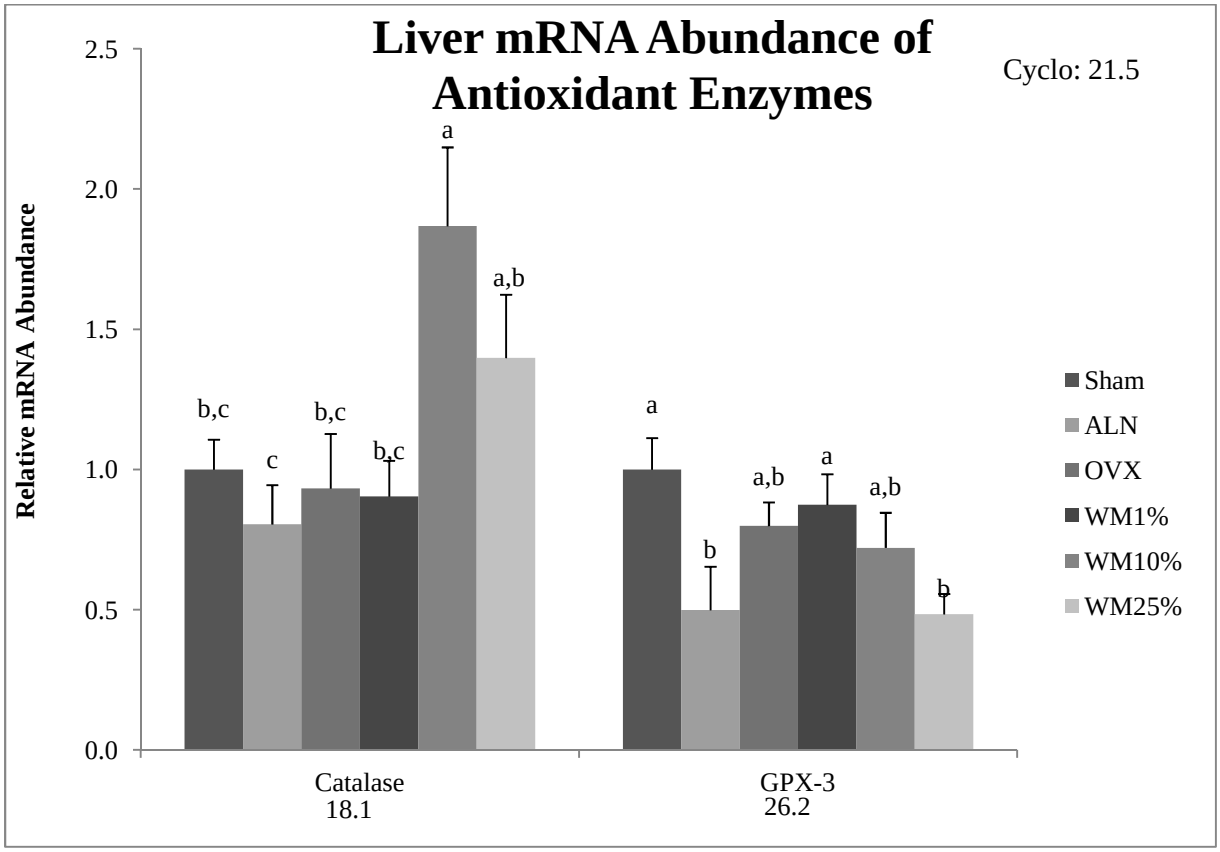

Figure 4. Comparison of liver relative mRNA abundance of antioxidant enzymes in OVX-ALN-control, OVX-Control, WM1%, WM105 and WM25% relative to Sham control group relative to Sham control group (set at 1.0). CT values for Sham control group are listed below the respective gene. Values are mean ± SEM; Bars that do not share the same superscript letter are significantly different ($p<0.05$). GPX-3= glutathione peroxidase 3. Cyclo= Cyclophilin was used as the invariant control and had a CT value of 21.5.

CHAPTER V

DISCUSSION

The purpose of the present study was to determine if watermelon, due to its antioxidant and anti-inflammatory properties, would be able to prevent bone loss in ovariectomized (OVX) mice. It has been shown in previous studies that this animal model is appropriate for studying postmenopausal osteoporosis (103, 104). Additionally, this animal model has been used for investigating the effects of dietary interventions in preventing or delaying post-menopausal bone loss or osteoporosis. Rendina *et al.* used an OVX model to demonstrate the effects of varying doses of dried plum on ovariectomy-induced bone loss and found that a high dose of dried plum supplementation prevented the loss of spine BMD and BMC (105). Mori-Okamoto *et al.*, used OVX mice to demonstrate that pomegranate extract was able to increase bone volume and trabecular number, and decrease trabecular separation (106). Studies have also been done using compounds found in fruits and vegetables such as hesperidin and sarsasapogenin to demonstrate their beneficial effects on bone parameters of OVX mice (107, 108). These studies are a few examples from the literature the show positive effects of dietary interventions on bone with an OVX mice model.

Our main objective was to determine if dietary watermelon supplementation would prevent the OVX-induced decrease on BMD, BMC, BMA and bone microarchitecture.

Watermelon was selected because it contains nutrients that have antioxidant and anti-inflammatory activity, including lycopene, vitamin C, β-carotene, citrulline, and cucurbitacin E (82, 83). Lycopene, vitamin C, and β-carotene have been shown to have antioxidant activity that improves bone health (84, 88, 97). Cucurbitacin E has also been implicated in the inhibition of oxidation in cells, but there is no direct link to bone health (94). Finally citrulline, an amino acid unique to watermelon, is a precursor for arginine, which is important in macrophages to mediate inflammatory responses (28). However, similar to cucurbitacin E, to our knowledge there is no research has been published that connects the mechanism of action citrulline to bone health. We hypothesized that these components working together could have a positive effect on bone and reduce bone loss in OVX mice.

We observed that there were no significant differences between the tibial and lumbar BMD of the OVX-control groups and the WM fed groups. Additionally, the whole body BMD values were significantly lower in the WM1% and WM25% groups compared to OVX-control, but WM 10% was comparable to that of OVX-control. The combined BMD values of tibia, lumbar and whole body suggest no possible benefit of watermelon in this parameter. A possible explanation for this could be that the weight gain in the WM 25% group due to increased adiposity, was counteracting any positive effects from the bioactive components in watermelon. Previous research has demonstrated that obesity increases inflammatory cytokine and oxidative stressors and could play a role in decreasing bone mass (109, 110). We also observed a significant increase in plasma glucose of the WM25% fed group, which could suggest insulin resistance as a result of the adiposity (111). WM1% may not have had enough watermelon to counteract the bone loss while WM25% group may have had too much of the watermelon, causing the increased adiposity. WM10% seemed to maintain more of a balance with the watermelon's positive and negative side effects. These results are consistent with other studies (124). Lucas *et.al.,* also found the lower dose of fruit supplementation more beneficial than a higher one. They discovered

that the high fat diet plus 1% mango showed higher whole body, tibial, and vertebral BMD compared to high fat alone, but high fat diet plus 10% mango did not produce these same effects (124).

The μCT analyses conducted also produced a similar trend that shows WM10% has a positive effect on a few of the bone parameters measured. Based on previous research, we predicted that the tibia and lumbar were shown to be practical sites to assess changes in bone parameters using μCT (104, 112). We observed slight positive outcomes with the WM10% fed groups in the lumbar trabecular bone, although the tibial trabecular and cortical bones were not affected. In women with osteoporosis, vertebral fractures are very common and generally occur below the mid-thoracic region (113). Nearly 3/4^{ths} of all vertebral fractures occur during routine daily activities, such as bending over and lifting objects, and only about 1/3rd of those come to medical attention and prompt preventative treatments (114). Analyses of the L4 region of the vertebra showed that the WM10% group was able to restore connectivity density to the levels of the sham-control group, despite the increase in body weight and body fat. As stated in the results, the WM10% group also had comparable BV/TV, structure model index, and trabecular separation as the sham control group; however, it was also comparable to the OVX-control group. This suggests that WM10% had intermediate effect in restoring these parameters. WM10% therefore might be the most suitable option for restoring bone parameters from the three doses administered.

In order to understand the mechanism by which WM10% had a positive effect on bone, anti-inflammatory and antioxidant markers were assessed in plasma and liver. Increased inflammation and oxidative stressors both have been associated with the onset and progression of postmenopausal osteoporosis (14, 16). We decided to observe systemic changes in these parameters so plasma was used to assess the activity of antioxidant enzymes, and liver mRNA was used to assess gene expression of both antioxidant enzymes and inflammatory cytokines.

The inflammatory cytokines chosen were TNF-α and IL-6, both of which increase osteoclastogeneis (14). Osteoclast production and activity is largely driven by cytokines, especially TNF-α, which works by increasing RANK-L production, increasing RANK in osteoclast precursors, as well as increasing responsiveness of RANK to RANK-L (9). Although IL-6 has receptors on both osteoblasts and osteoclasts, its main physiological effects occur through other cytokines. IL-6 stimulates IL-1 release that increases osteoclast formation as well as mediates the stimulatory effects of TNF-α on osteoclast precursors (31). We did not see any significant changes with the inflammatory cytokines, TNF-α and IL-6, in liver mRNA abundance. One of the reasons this could have occurred was because there was a lot of variability with the TNF-α data in each group. Although not significant, IL-6 did not display this variation and mRNA levels seemed to decrease in the WM fed groups.

We also assessed systemic antioxidant expression in plasma and liver. Lean and colleagues demonstrated that the administration of antioxidants, ascorbate or N-acetyl- cysteine (precursor to glutathione), prevented OVX- induced bone loss in mice (115). Similarly, Rao *et. al.,* found that lycopene intake in postmenopausal women decreased protein oxidation and lipid peroxidation while also decreasing bone resorption (26). Catalase and GPX are both responsible for neutralizing reactive oxygen species, specifically hydrogen peroxide, which is essential for bone loss due to estrogen deficiency and osteoclastogenesis (116). Furthermore, GPX-3 is highly abundant in the plasma, and therefore liver mRNA abundance of GPX-3 specifically was observed (117). Liver Catalase mRNA was significantly higher in the WM10% fed groups compared to all other groups and was comparable to the WM25% group. However when analyzing the plasma we observed no significant difference in catalase and GPX activity between the groups. These results were consistent with the results from our bone analyses, suggesting that WM10% may be the optimal done to prevent bone loss through increased antioxidant activity.

In the current study, we demonstrated that our OVX model had increased bone loss as evidenced by decreased BMD and compromised μCT architecture. The OVX model is also expected to gain body weight as supported in other studies (103, 118). However in the present study, the OVX mice fed 10% and 25% watermelon diets had significantly increased body weights and body fat, by the completion of week 1, compared to OVX-control. Food intake remained similar throughout the study between the groups and diet compositions were isocaloric, isonitrogenous and had the same calcium and phosphorus concentrations. Possible explanations for this change in body composition could be related to the components in the watermelon altering the gut microbiota that could possibly be promoting weight gain. Greiner *et.al*, have demonstrated that alterations in gut microbiota can increase obesity and promote insulin resistance (119). Additionally, Laparra *et.al*, have discussed that phytochemicals from functional foods that are used by the bacteria to produce secondary metabolites, are also able to influence growth and activity of specific intestinal bacteria (120). This leads to the possibility that there is an alteration of gut bacteria in the C57BL/6 female mice through the bioactive components of watermelon, especially those that exist in a larger quantity such as lycopene. A study done by Kim *et.al*, observed similar effects and found that with increased doses of lycopene, there was increased weight gain in female B6C3F1 mice, a cross between C57BL/6 and C3H mice, however in a chemoprevention study rather than an OVX model (121). These explanations on the effect of watermelon on body weight and composition are all speculative and warrants further investigation.

The weight gain observed in the watermelon-fed mice prompted us to examine lipid parameters. As expected all OVX groups had increased proportions of fat in the liver and sham control had the lowest which was comparable to watermelon 10% and 25%. This suggests that watermelon prevented the accumulation of fat in the liver. When feces were analyzed, WM groups had the highest fecal fat content, however, this was not significantly different from ALN-

control. Previous research has shown that ovariectomy causes hyperlipidemia (104, 122), however we did not observe that in our study with our plasma cholesterol analysis. Although not significantly different, the WM fed groups seemed to have lower liver cholesterol compared to the OVX and sham, which points towards another possible benefit of watermelon, but this needs to be further explored.

Limitations

Although WM10% seems promising, it is necessary to keep in mind the limitations of this study. Bones could have also been used to assess antioxidant enzymes to observe a more local effect of the watermelon rather than using liver. Lean *et.al.*, measured glutathione and glutathione reductase in OVX mice, and found that levels were unchanged in liver and spleen compared to sham, however, they were decreased in the bone marrow (115), which may explain why glutathione peroxidase was not significantly different in liver mRNA expression and plasma activity. Another limitation is that the data obtained from an animal model may not be translatable to humans. The mouse genome is about 85% similar in sequence to the human genome, but the 15% difference could translate into countless differences (122). Additionally, the *Mus musculus* (house mouse) only has a lifespan of about two to three years in a protected, well-controlled environment (123). The mouse model should be used as a basis for furthering research with watermelon and conducting a human study as the next step.

Conclusion

In conclusion, our data indicates that watermelon, in a moderate amount, might be a suitable dietary option for maintaining skeletal health. Possible components in watermelon that could be responsible for these benefits include lycopene, vitamin C and beta-carotene as antioxidants, and citrulline and cucurbitacin E as anti-inflammatory agents. There is an unknown mechanism by which watermelon is increasing weight gain in OVX mice, which needs to be

further explored. Such research could include experiments on the components in watermelon, such as lycopene, and alterations in gut microbiota of OVX mice that are influencing weight gain, and if that would also exhibit the same effect in humans. In this study, we found that with high doses of watermelon, the beneficial effects of the bioactive components could possibly be counteracted by increased inflammation and oxidation due to the increased adiposity. A possible way to explore this would be to design a WM5% and WM15% diet and examine if the effects have a range in which they provide maximum benefit, and examine other bones in the OVX mice to see if the watermelon is exhibiting its effects elsewhere. Finally, watermelon could also possibly have an effect of reducing cholesterol in OVX mice as it was observed in this study, despite the increased body weight, and the follow up experiment could also take into consideration circulating cholesterol levels. Although more research is needed in this area, watermelon in moderate amounts, along with a variety of other fruits and vegetables, should be included in the diet of a postmenopausal woman to aid in the process of slowing down bone loss.

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APPENDICES

Oklahoma State University
Institutional Animal Care and Use Committee (IACUC)

Protocol Expires: 8/5/2015

Date : Monday, August 06, 2012 Animal Care and Use Protocol (ACUP) No : HS122

Proposal Title: Freeze-dried watermelon in preventing postmenopausal osteoporosis

Principal
Investigator:

Edralin Lucas
Nutritional Sciences
301 HS
Campus

Reviewed and Processed as: Full Committee

Approval Status Recommended by Reviewer(s) : Approved

The revised protocol is approved. You are approved to use a maximum of 90 mice for the next three years.

Signatures :

Charlotte Ownby, IACUC Chair

Monday, August 06, 2012
Date

cc: Department Head, Nutritional Sciences
Director, Animal Resources

Approvals are valid for three calendar years, after which time a request for renewal must be submitted. Any modifications to the research project, course, or testing procedure must be submitted for review and approval by the IACUC, prior to initiating any changes. Modifications do not affect the original approval period. Approved projects are subject to monitoring by the IACUC. OSU is a USDA registered research facility and maintains an Animal Welfare Assurance document with the Public Health Service Office of Laboratory Animal Welfare, Assurance number AA3722-01.

VITA

Maryam Mahmood

Candidate for the Degree of

Master of Science

Thesis: THE EFFECTS OF FREEZE-DRIED WATERMELON ON BONE AND CLINICAL PARAMETERS OF OVARIECTOMIZED MICE

Major Field: Nutritional Sciences

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Completed the requirements for the Master of Science in Nutritional Sciences at Oklahoma State University, Stillwater, Oklahoma in May, 2014.

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